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<p>(21) International Application Number: PCT/US90/00191 (22) International Filing Date: 8 January 1990 (08.01.90) (30) Priority data: 297,501 13 January 1989 (13.01.89) US (71) Applicant: BIO-RAD LABORATORIES, INC. [US/US]; 1000 Alfred Nobel Drive, Hercules, CA 94647 (US). (72) Inventor: HJERTEN, Stellan ; Langvagen 7B, S-752 52 Uppsala (SE). (74) Agent: HEINES, M., Henry; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).</p>		<p>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published With international search report.</p>
<p>(54) Title: CHROMATOGRAPHY ON A CONTINUOUS POLYMER</p> <p>(57) Abstract</p> <p>Macromolecular species in a sample are chromatographically separated in a manner analogous to that which would occur in molecular sieve chromatography (gel filtration), by being passed through a separation medium comprised of a non-water-soluble fluid-permeable plug of cross-linked polyacrylamide, where the degree of cross-linking is sufficient to cause discontinuities, gaps or channels in the polymer network large enough to permit water and the macromolecules in the sample to pass through, and yet interfering with the free flow of the macromolecules sufficiently to cause their bulk flow rate through the plug to vary with their molecular weights. By varying the nature and composition of the monomers, the plug can be designed alternatively for a wide variety of types of chromatography, including ion exchange, hydrophobic interaction, affinity, boronate and dye-ligand chromatography.</p> <p style="text-align: center;">BEST AVAILABLE COPY</p>		

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CHROMATOGRAPHY ON A CONTINUOUS POLYMER

5 This invention relates to chromatographic separations based on common chromatographic properties such as molecular size, charge, hydrophobicity, and bioaffinity and also to chromatographic separation media useful for such separations.

10

BACKGROUND AND SUMMARY OF THE INVENTION

Conventional chromatography involves the passage of a sample through a bed of beads. It is not possible, however, to achieve a perfectly packed bed. Heterogeneities
15 in the packed bed give rise to zone broadening, which is a disturbing factor. Further disadvantages of packed beds are the time-consuming and expensive steps required for preparation of the beads, the sieving of the beads to select the desired size, and the packing of the column with the
20 beads.

It has now been discovered that these disadvantages can be avoided by the use of a chromatographic separation medium in the form of a continuous, coherent gel plug formed from monomers which upon polymerization form a
25 structure which has channels large enough to permit the passage of an eluent, and properties rendering it useful for such separation techniques as molecular sieve, ion exchange, hydrophobic interaction, affinity, boronate, and dye ligand chromatography. Such channels may be formed, for instance,
30 when the monomers include species which are of limited solubility in water. When these monomers are used in sufficient amounts or proportions, the resulting polymers have a sufficiently hydrophobic character to cause them to precipitate out of solution. As a result of this
35 precipitation, the polymer chains tend to adhere to each other in bundles, with the voids or channels referred to above forming between the bundles. One example of a monomer

meeting this description is N,N'-methylenebisacrylamide, and preferred polymers in accordance with the invention are polyureas, particularly polyacrylamides.

5 The highly homogeneous structure of the gel plug produces separation of eluents into very narrow zones, and the plug is easily and reproducibly prepared directly in the capillary, column or other enclosure in which the chromatographic separation is to take place, by simply filling the enclosure with monomer solution.

10 Further features and advantages of the invention will become apparent from the description which follows.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

15 The continuous, channel-permeated structure to be described herein is one which results from a degree of cross-linking which is sufficient to both render an otherwise transparent polyacrylamide gel opalescent, i.e., to make it less water-soluble. The degree of cross-linking
20 will also be sufficient to draw the polymer chains close enough together to form cracks or channels in the polymer network large enough to permit the passage of water and the sample component molecules. The continuous structure adjacent to the cracks may be porous or nonporous.

25 In general, the formation of these channels will be achieved by using a mole ratio of cross-linking agent to linear-chain-forming monomer of at least about 0.45, preferably at least about 0.55, and most preferably at least about 0.70.

30 The monomers used to form the polymer will be selected in accordance with the type of chromatography for which the plug is intended to be used. The molecular structure of the monomer and the selection of functional groups on the monomer which will be appropriate for any
35 particular type of chromatography will be readily apparent to those skilled in the art. One may thus for example design a plug within the present invention suitable for use

in molecular sieve chromatography, ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, boronate chromatography, or dye ligand chromatography.

5 Preferred linear-chain-forming monomers are acrylamides and other derivatives of acrylic acid. Acrylamide itself is a particularly preferred linear-chain-forming monomer, which may be used alone or in combination with other structurally related species such as the methyl
10 ester of acrylic acid. A preferred cross-linking agent, as mentioned above, is N,N'-methylenebisacrylamide, and for systems where the linear-chain-forming monomer is acrylamide, the mole ratios given above are equivalent to C values (weight percent of cross-linker to total of cross-
15 linker and linear-chain-forming monomer) of 50% or more, 55% or more, and 60% or more.

In the preferred embodiment of the invention in which the polymer is a polyacrylamide, any of the wide variety of cross-linking agents known in the art for use
20 with acrylamide monomer may be utilized. Included among such agents are bisacrylamides, diacrylates, and a wide range of terminal dienes. Specific examples are dihydroxyethylenebisacrylamide, diallyltartardiamide, triallyl citric triamide, ethylene diacrylate,
25 bisacrylylcystamine and N,N'-methylenebisacrylamide.

The plug is formed by polymerization according to conventional techniques well known among those skilled in the art. The plug may for example be formed from an aqueous solution of the monomer and cross-linking agent and polymer-
30 ization catalysts and other conventional additives directly in the casing or column tube in which the plug is intended to be used. It will be advantageous in such cases to covalently bind the plug to the inner wall of the column. This may be achieved by binding agents, such as for example
35 Bind Silane, according to conventional techniques. Aqueous solutions in which the polymer components comprise from about 1% to about 20% by weight of the solution are

preferred. Within this range, concentrations of about 2% to about 10% are further preferred, and concentrations of about 2.5% to about 5% are the most preferred.

5 Separation of a sample into its components is likewise achieved in accordance with conventional techniques. Water, or preferably a buffer solution with a pH of about 7.0 to about 8.5, will preferably serve as a carrier for the sample through the plug, and flow may be achieved by pumping or gravity flow. Detection of the separated sample components may then be achieved by conventional means, 10 either in the column itself, using staining methods if necessary, or downstream of the plug or outside the column as the components elute individually from the column. Separation media of this type are particularly effective for the separation of species having molecular weights ranging 15 from about 1000 to about 1,000,000.

The following examples are offered strictly for purposes of illustration, and are intended neither to limit nor to define the invention in any manner.

20

EXAMPLES

A glass wool plug was placed in a Pasteur pipette at the top of the constricted region of the pipette. A length of plastic tubing fitted with a hose clamp was 25 secured to the pipette tip.

A buffer solution containing 0.01 M Tris-HCl and 10% (weight/volume) sucrose at pH 7.5 was poured into the pipette. The hose clamp was then opened. When the buffer level reached the glass wool, the hose clamp was closed to 30 stop the buffer flow.

A deaerated mixture was then poured into the pipette and allowed to polymerize. The mixture consisted of 1 mL of an aqueous solution of acrylamide and N,N'-methylenebisacrylamide (at concentrations and proportions listed 35 below), 6 μ L of a 10% (weight/volume) solution of ammonium persulfate, and 1 μ L of tetramethylethylenediamine.

Once the solution polymerized, it formed a non-water-soluble continuous structure spanning the width of the pipette above the glass wool. A sample consisting of the following components was then applied to the top of the polymer structure:

	phycoerythrin	M.W. 290,000
	phycocyanin	M.W. 135,000
	cytochrome	M.W. 13,000
10	bromophenol blue	M.W. 1,000

A buffer solution consisting of 0.01 M Tris-HCl, pH 7.5, was layered above the sample, and the sample and buffer solution were permitted to flow through the pipette by gravity flow.

15 A variety of polyacrylamide compositions and buffer solution flow rates were used, as listed in the table below. In this table, the symbol T designates the concentration in weight percent of the acrylamide and N,N'-methylenebisacrylamide combined in the forming solution, the symbol C designates

20 the proportion of N,N'-methylenebisacrylamide to the combination of acrylamide and N,N'-methylenebisacrylamide expressed in weight percent, and the last column represents the flow rates in values relative to each other for each C value. The flow rate given for T = 4, C = 60 is equivalent

25 to about 4-5 hours for the entire elution. Direct comparisons can be made among flow rates for the various C values with a single T value.

	<u>Gel Composition</u>		<u>Gravity Flow</u>
	<u>T (%)</u>	<u>C (%)</u>	
30	4	60	0.8
	4	50	0.3
35	4	40	<0.3
	4	30	<0.3
40	4	20	0

	<u>Gel Composition</u>		<u>Gravity Flow</u>
	<u>T (%)</u>	<u>C (%)</u>	
5	3	60	2.5
	3	50	1.8
	3	40	<1.8
10	3	20	0
15	2	60	3
	2	50	5
	2	30	0
20	20	30	0
	20	15	0
25	10	30	0
30	6	10	0
	6	20	0
	6	30	0

35 In those runs where a positive gravity flow was detectable, the four components listed above separated into discrete bands, in order of increasing molecular weight, with the component of highest molecular weight (phycoerythrin) showing the greatest mobility through the column.

40

The foregoing is offered primarily for purposes of illustration. It will be readily apparent to those skilled in the art that modifications and substitutions in terms of the materials, procedures and other parameters of the system may be introduced without departing from the spirit and scope of the invention.

45

WHAT IS CLAIMED IS:

1. A chromatographic column containing a separation medium comprising a non-water-soluble plug
5 spanning the cross-sectional area of said column, said plug comprised of a cross-linked polymer having a sufficient degree of cross-linking and being sufficiently hydrophobic to form channels permeable to macromolecular eluents.
- 10 2. A chromatographic column in accordance with claim 1 in which said cross-linked polymer is formed from a mixture of a linear-chain-forming monomer and a cross-linking agent in which the mole ratio of cross-linking agent to the total of linear-chain-forming monomer and cross-
15 linking agent is at least about 0.45.
3. A chromatographic column in accordance with claim 1 in which said cross-linked polymer is formed from a mixture of a linear-chain-forming monomer which includes
20 acrylamide and a cross-linking agent which is a bisacrylamide.
4. A chromatographic column in accordance with claim 1 in which said cross-linked polymer is formed from a
25 mixture of a linear-chain-forming monomer which includes acrylamide and a cross-linking agent which is N,N'-methylenebisacrylamide, said N,N'-methylenebisacrylamide comprising at least about 50% by weight of the total of said acrylamide and said N,N'-methylenebisacryla-
30 mide.
5. A method of separating a mixture of macro-
molecular species in a liquid sample into components on the basis of molecular size, said method comprising passing said
35 sample through a chromatographic column containing a separation medium comprising a non-water-soluble plug spanning the cross-sectional area of said column, said plug

comprised of a cross-linked polymer having a sufficient degree of cross-linking and being sufficiently hydrophobic to form channels permeable to macromolecular eluents, to cause said components to separate into substantially
5 discrete bands within said plug.

6. A method in accordance with claim 5 in which said cross-linked polymer is formed from a mixture of a linear-chain-forming monomer and a cross-linking agent in
10 which the mole ratio of cross-linking agent to the total of linear-chain-forming monomer and cross-linking agent is at least about 0.45, and said plug when saturated with water comprises from about 1% to about 20% by weight of said plug and said water combined.

15 7. A method in accordance with claim 5 in which said cross-linked polymer is formed from a mixture of a linear-chain-forming monomer which includes acrylamide and a cross-linking agent which is N,N'-methylenebisacrylamide.

20 8. A method in accordance with claim 5 in which said plug is saturated with a buffer solution at a pH of about 7.0 to about 8.5, and said plug comprises from about 2.5% to about 5% by weight of said plug and said
25 water combined.

9. A method in accordance with claim 5 in which said passing sample through said plug is achieved by gravitational flow.




30 10. A method of separating a mixture of macromolecular species in a liquid sample into components on the basis of molecular size, said method comprising passing said sample through a chromatographic column comprising a non-
35 water-soluble fluid-permeable plug of cross-linked polyacrylamide formed from a mixture of acrylamide and N,N'-methylenebisacrylamide, said N,N'-methylenebis-

acrylamide comprising at least about 55% by weight of the total of said acrylamide and said N,N'-methylenebisacrylamide, and said plug when saturated with water comprising from about 2.5% to about 5% by weight of said plug and said

5 water combined.

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US90/00191**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5):B01D 15/08 U.S. Class 210/635,656,198.2,502.1;55/386																	
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">U.S.</td> <td style="padding: 5px;">55/386 210/635,656,198.2,502.1</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	U.S.	55/386 210/635,656,198.2,502.1											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category [*]</th> <th style="border-bottom: 1px solid black;">Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 15%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">US, A, 3,878,092 (FULLER) 15 April 1975, see column 7, lines 53-59 and column 8, lines 45-52</td> <td style="vertical-align: top; padding: 5px;">1-10</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">US, A, 3,808,125 (GOOD) 30 April 1974, see column 6, lines 25-34 and column 7, lines 13-19</td> <td style="vertical-align: top; padding: 5px;">1-10</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">US, A, 4,793,920 (CORTES) 27 December 1988, see column 5, lines 18-39 and column 2, lines 29-36</td> <td style="vertical-align: top; padding: 5px;">1-10</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">Mikes, Laboratory Handbook of Chromatographic and Allied Methods published 1979, by Ellis Horwood Limited see pages 335,336,343-347, and 412-414, especially page 343, lines 1-10</td> <td style="vertical-align: top; padding: 5px;">1-10</td> </tr> </table>			Category [*]	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	US, A, 3,878,092 (FULLER) 15 April 1975, see column 7, lines 53-59 and column 8, lines 45-52	1-10	Y	US, A, 3,808,125 (GOOD) 30 April 1974, see column 6, lines 25-34 and column 7, lines 13-19	1-10	Y	US, A, 4,793,920 (CORTES) 27 December 1988, see column 5, lines 18-39 and column 2, lines 29-36	1-10	Y	Mikes, Laboratory Handbook of Chromatographic and Allied Methods published 1979, by Ellis Horwood Limited see pages 335,336,343-347, and 412-414, especially page 343, lines 1-10	1-10
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">26 February 1990</td> <td style="border-bottom: 1px solid black; padding: 5px; text-align: center;">28 MAR 1990</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px;">ISA/US</td> <td style="padding: 5px; text-align: center;">  Ernest G. Therkorn </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	26 February 1990	28 MAR 1990	International Searching Authority	Signature of Authorized Officer	ISA/US	 Ernest G. Therkorn							
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